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A Defined Substrate Technology for the Enumeration of Microbial Indicators of Environmental Pollution

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The examination of water and other environmental sources for microbial pollution is a major public health undertaking. Currently, there are two accepted methods in use: the multiple-tube fermentation (MTF) and the membrane filtration (MF) tests. Both methods are designed to enumerate the secondary indicator group, total coliforms. Both tests suffer several inherent limitations, including a time delay of three to seven days to obtain a definitive result, the subjective nature of the test interpretation, and the inability to provide directly useful public health information. A defined substrate technology, originally used to enumerate specific bacterial species from mixtures in clinical urine specimens, was applied to water testing; the technology was constituted to enumerate simultaneously both total coliforms and the primary indicator bacterium *E. coli*. Examination of environmental isolates of these two classes of target microbes showed sensitivity equal to available methods, with potentially greater specificity. It was not subject to inhibition by bacteria other than the targets, grew injured coliforms, did not require confirmatory tests, and the maximum time to a positive was 24 hours. The defined substrate technology provides both regulatory and directly useful public health information.

INTRODUCTION

The testing of water and other environmental sources for microbial pollution dates to the 1880s, when Escherich identified the bacterium *Bacillus coli* (now *Escherichia coli*) and established it as always present in the feces of warm-blooded animals. He later recommended the analysis for this bacterium as a test for the acceptability of water for human consumption [1]. The presence of this species was considered a sentinel for the primary pathogens *Salmonella, Shigella,* viruses, and the like, which were difficult to isolate. The use of *E. coli* as the main microbial indicator was modified because it was laborious and time-consuming to isolate and identify this bacterium from the mixture of microbes present in environmental samples. Thus, we now enumerate an *E. coli* surrogate group, the total coliforms, as the means of determining if water is free of microbial pollution. The total coliform group is defined as lactose-positive *Enterobacteriaceae* [2]; this group includes the former paracolon bacilli, which is composed primarily of *Citrobacter* species and anaerogenic *E. coli*. The broad total coliform group was adopted because its members were relatively easy to enumerate and were present in greater numbers than the pathogens they were thought

Abbreviations: CFU: colony-forming unit HPC: heterotrophic bacteria MF: membrane filter MPN: most probable number MTF: multiple-tube fermentation MUG: 4-methyl-umbilliferyl-β-D-glucuronide O.D.: optical density ONPG: ortho-nitrophenyl-β-D-galactopyranoside

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to accompany [3,4]. They are not in themselves pathogenic and provide no useful direct evidence of the presence of pathogenic microbes.

Routine testing for coliforms in potable water was instituted in the 1920s [2]. The method developed was the multiple-tube fermentation (MTF). Ten milliliters of water were added to each of five test tubes containing a protein base with the fermentable carbohydrate lactose. After 48 hours' incubation, the number of coliforms per 100 ml was calculated from the number of tubes positive (most probable number or MPN). The MPN value was an estimate of the number of bacteria in a sample based on the number of tubes positive/number of tubes inoculated [2]. Because the method was subject to false-positives, additional validation tests had to be performed. To complete an entire MTF analysis now requires between two and six days. At each step, the determination of positivity may be highly subjective because one must sometimes discriminate among subtle differences in the final end products. A MTF test does not yield a species identification but only a positive or negative for that individual tube. Public health laws were written so that a water analysis was considered satisfactory if no more than one tube of the five inoculated with the water sample was positive; water was considered safe for consumption if it met this standard [2].

In the 1950s, the membrane filtration (MF) technique was developed [5]. A given volume of water, generally 100 ml, was passed through a bacterial exclusion filter (0.45 μm membrane). The membrane was placed on the surface of an agar medium containing a protein base, lactose as the fermentable carbohydrate, and a pH indicator. Total coliforms demonstrated green "sheen" colonies. Like the MTF technique, confirmation and completed steps must be performed because the primary plate can yield a false-positive result [6]. Also, like the MTF method, no useful public health information was obtained from an analysis; the test was used for regulatory purposes only. Regulations allow a maximum of four total coliforms per 100 ml in any one sample or an average of one per 100 ml for a month [2,7,8,9].

Until the 1970s, the available means of analysis served public health agencies well. If utilities saw increasing numbers of coliforms, they increased the amount of chlorine disinfectant in the system to eliminate them. During this decade, two phenomena were noted that were to affect testing profoundly. First, it was found that chlorine reacted with organics in water to form carcinogenic trihalomethanes; therefore, utilities had to lower the average chlorine concentration in water and could not easily raise it. Second, an aging infrastructure created an environment whereby coliforms could colonize water distribution systems and be continuously present. Many systems began to experience permanent coliform biofilm occurrences, which made the employment of these bacteria as sentinels of pathogens useless and created a dilemma for public health authorities [10,11].

Both MTF and MF media are based on the same principle of microbial selection. Each includes a broad protein base that allows the growth of virtually all aerobic bacteria. Detergents are added to inhibit yeasts and gram-positive bacteria from growing. The coliforms are further differentiated from other bacteria by the fermentation of lactose with the resultant decrease in pH and change in color of the medium in the MTF tube or colony on MF agar [12]; however, these ingredients are only relatively effective, and the MTF and MF methods have demonstrated major sensitivity and specificity limitations. The sensitivities of both methods are significantly affected by the presence of gram-negative bacteria other than coliforms [12,13,14]. Furthermore, coliforms themselves may not produce enough acid to yield a pH change in the

media [15,16]. The specificity of both methods has been shown to vary between 70 and 85 percent, primarily because the activity of the inhibitors is not absolute [17,18,19].

A defined substrate method was developed to overcome the limitations of the MTF and MF methods and at the same time to provide direct public health information. The new technique is a modification of technology used to enumerate and identify urinary tract pathogens [20]. The species of microbes most commonly isolated from water and water distribution systems are the same as the species isolated from human urinary tract infections. These include *E. coli, K. pneumoniae, E. cloacae,* and *C. freundii.* Unlike the MTF and MF methods, which grow all aerobic microbes and eliminate non-coliforms with inhibitory chemicals, the defined substrate technology is based on the principle that one feeds only the target microbe(s) (here, total coliforms and *E. coli*) and does not provide sustenance for other bacteria. Therefore, only these microbes grow; one does not have to add inhibitors to eliminate other bacteria. A defined substrate is used as a vital nutrient source for the target microbe(s). During the process of substrate digestion, a chromogen is released from the defined substrate, indicating the presence of the target(s) [21,22,23].

The defined substrate technology used for water analysis employs the substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG) for the constitutive enzyme β-galactosidase, present in all total coliforms. In addition, a second defined substrate, 4-methyl-umbilliferyl-β-D-glucuronide (MUG), is used specifically for *E. coli.* Since ammonium sulfate is the only source of nitrogen, a unique feature of the defined substrate technology is that the metabolic activity of the target microbe is directed toward the substrate(s). Because microbes other than the target(s) cannot grow and metabolize, there is no need for additional tests after a color change specific for the target(s) has been observed.

The defined substrate technology was applied to water analysis and tested to delimit its sensitivity and specificity. Particular attention was paid to determine if heterotrophic bacteria (HPC) other than total coliforms and *E. coli* could yield a false-positive test or if HPC could suppress the targets to produce a false-negative analysis. Heterotrophic bacteria are those that require formed nutrients in order to grow. In addition, the sensitivity of the method in enumerating injured coliforms was examined.

MATERIALS AND METHODS

Bacterial Isolates

Environmental isolates of *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae,* and *Citrobacter freundii* were obtained from Lake DeForest, New City, New York. They were identified as to species by commonly accepted methods [24].

Defined Substrate Method

The defined substrate method was dispensed into 13 × 100 mm test tubes. It contained, per liter: $(NH_4)_2SO_4$, 5 g; $Mn(SO_4)_2$, 500 mcg; $ZnSO_4$, 500 mcg; $MgSO_4$, 100 mg; NaCl, 10 g; $CaCl_2$, 50 mg; KH_2PO_4, 900 mg; $NaPO_4$, 6.2 g; Na_2SO_3, 40 mg; Amphotericin B, 1 mg; Tween 80; 50 mg; ortho-nitrophenyl-β-D-galactopyranoside, 500 mg (ONPG); and 4-methyl-umbilliferyl-β-D-glucuronide (MUG), 75 mg. Each test tube received 1 ml. ONPG served as the defined substrate for total coliforms and MUG for *E. coli.* Sodium sulfite was utilized as an agent to assist in the repair of

bacterial cell walls. Amphotericin B was used to inhibit yeasts. The non-ionic detergent Tween 80 was utilized as dispersing agent; it had no anti-bacterial activity. All ingredients were obtained from the Sigma Chemical Co. (St. Louis, MO).

The defined substrate method was constituted as a MPN test, with each tube receiving 10 ml of sample. The defined substrate tube was colorless after the bacterial suspension had been added. The tubes were incubated in ambient air at 35°C. Any yellow in the test tube was taken as a positive for total coliforms. Each yellow tube was exposed to a four-watt, 366 nm light (UVP, Inc., San Gabriel, CA); blue-white fluorescence demonstrated the presence of *E. coli*. No additional confirmatory tests need be performed. The number of bacteria per 100 ml was determined by the number of tubes positive from standard MPN tables [2]. In order to determine the relationship between the visible estimate of color production (4+, 3+, and so on) and the actual amount of color generated, test tubes were examined in a spectrophotometer able to accept them directly (Bausch & Lomb, Buffalo, NY), with optical density measured at 445 nm.

Sensitivity

Sensitivity was determined by growing each of the environmental total coliforms in trypticase soy broth overnight and diluting them in sterile tap water to a final suspension of 32, 16, 8, 4, 2, and 1 colony-forming units (CFU) per 100 ml. The concentration of bacteria was confirmed by filtering 500 ml of bacterial suspension through a 22 μm membrane (Millipore Corp., Waltham, MA) and placing it on a plate count agar plate. Defined substrate tubes were inoculated with each concentration of test isolate, with the number of tubes used for each dilution determined by the standard MPN table [2]. Each bacterial concentration was repeated three times.

After inoculation, all tubes and plates were incubated at 35°C in ambient air. All defined substrate tubes were inspected visually for the development of a yellow color at 12, 14, 16, 18, 20, 22, and 24 hours. In addition, an optical density (O.D.) reading was made at 445 nm. Any tube showing a yellow color was examined for fluorescence by exposing it to 366 nm light.

Specificity

In order to determine if bacteria other than coliforms or *E. coli* could affect the test, heterotrophic bacteria were mixed with them in ratios of 2×10^6:1 to 325:1. HPC bacteria were obtained from Lake DeForest; they were identified by commonly accepted procedures [25,26]. Like the coliform bacteria, they were grown in trypticase soy broth for 18 to 24 hours and then diluted in sterile tap water. Final concentrations of HPC were 20,000, 10,000, 5,000, 2,500, 1,250, 625, and 325 per ml. Bacteria tested included an *Aeromonas hydrophilia, Flavobacterium breve,* and *Pseudomonas maltophilia.* The *Aeromonas hydrophilia* and *Pseudomonas maltophilia* contained the β-galactosidase system but appeared to lack the permease cascade to bring the ONPG substrate into the cell. These two species, which can produce false-positive MTF and MF tests, provided a means of determining if false-positive defined substrate analyses would occur at high bacterial concentrations. All heterotrophs were mixed with each of the coliform species so that the final defined substrate tube contained each concentration of HPC with four coliforms per 100 ml. Incubation, analysis, and interpretation of the test proceeded as described in the section on Sensitivity.

Injured Coliform Analysis

Bacteria injured by chlorine may be difficult to detect by available methods. In order to determine if the defined substrate method could enumerate these bacteria, the four species of coliforms were injured according to the method of McFeters and Stuart [27]. Briefly, coliforms were grown for 18 to 24 hours in trypticase soy broth and washed twice in sterile distilled water. The coliform was resuspended to 10^9 bacteria per ml. Fifty ml of the bacterial suspension were placed in a membrane dialysis bag and immersed for one minute in a sodium hypochlorite solution adjusted to yield a final chlorine concentration (determined by the DPD method, Hach Chemical Co., Ames, IA) of 0.5 ppm (0.5 mcg per ml). Residual chlorine was immediately inactivated by the addition of sodium thiosulfate. As a control, the same coliforms were treated in parallel, except that they were not exposed to chlorine. To insure that the coliforms were injured, subcultures from the chlorine-treated dialysis bags were made on to tryptic soy broth with lactose and yeast extract (to count repairable cells) and the same agar with 0.1 percent sodium desoxycholate (to count lethally damaged cells). Treated and untreated bacteria from the dialysis bags were diluted in sterile tap water and enumerated by the defined substrate method, as described in the section on Sensitivity. The ability to recover injured coliforms was determined by comparing the number of positive defined substrate tubes and the time to positivity from both chlorine-treated and untreated populations.

RESULTS

The defined substrate technology demonstrated the sensitivity of one CFU per 100 ml expected by MTF and MF methods and required by public health authorities. As Fig. 1 shows, each of the four test species of environmental coliforms was detected within 24 hours at this level. Once the minimum amount of color had been seen, at an O.D. of approximately 0.03 at 445 nm, the bacteria appeared to be in log phase. Increases in color intensity were rapid with maximum absorbency achieved within an additional four to six hours of incubation. At one CFU per 100 ml, each of the species did not achieve a color intensity of 4+ (O.D. > 1.5); however, each species was distinctly visible (color intensity of 2+, O.D. $= 0.6$ or more). The greater the initial concentration of bacteria, the earlier was positivity noted. At a concentration of 32 bacteria per 100 ml, the yellow color could be seen after 14 hours' incubation; at 16 bacteria per 100 ml, positive tubes were seen at 16 hours.

Fluorescence produced by *E. coli* could also be detected within 24 hours at one CFU per 100 ml and within 14 hours at 32 CFU per 100 ml. Since this bacterium provides a direct measure of fecal contamination, useful information of direct public health importance was obtained simultaneously with the regulated total coliform group. None of the other species tested produced fluorescence.

A serious limitation for the enumeration of small numbers of coliforms by the MTF and MF methods is the competition they face for nutrients from heterotrophic bacteria. As in any confined ecological niche (i.e., a tube of broth, the surface of agar) those bacteria that utilize nutrients most efficiently will grow at the expense of those that are less able to compete. In nature there is a ratio of from 500:1 to 5×10^6:1 of HPC to coliforms. As Table 1 shows, the defined substrate technology was refractory to the inhibition of high concentrations of the three species of heterotrophs tested.

Mixtures of the HPC also did not result in false-negative analyses. When HPC were

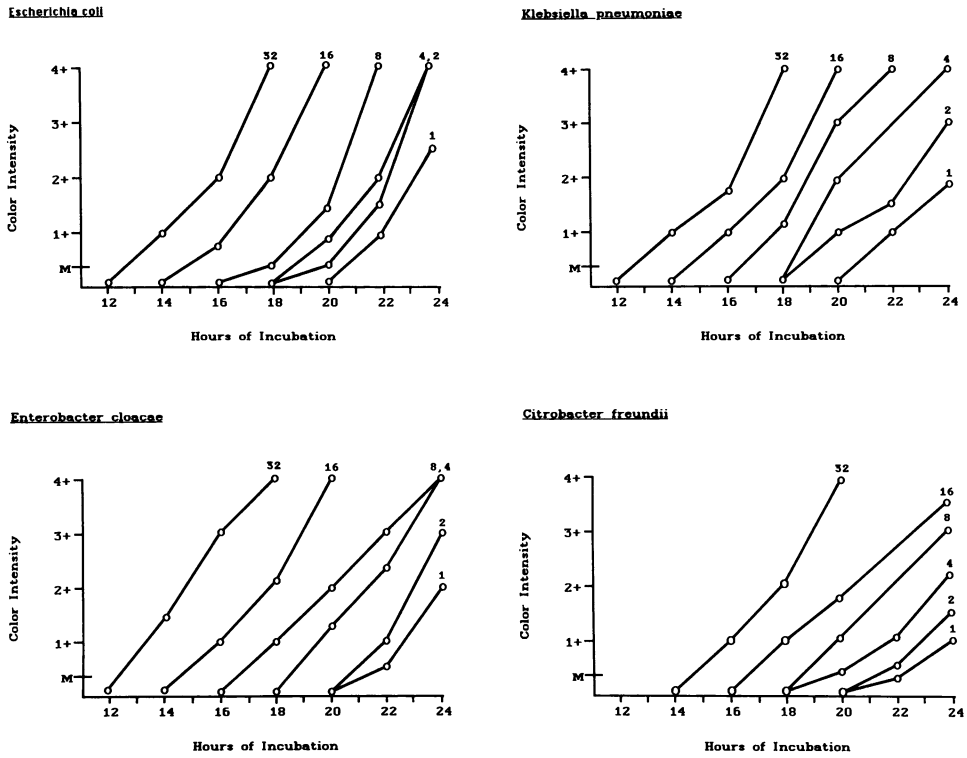

FIG. 1. The generation of the ortho-nitrophenyl chromogen by each of the four species of total coliforms as a function of bacterial inoculum and time. Each coliform was in pure culture. The numbers above the last reading represent the starting CFU per 100 ml. **M**: the minimum detectable visible color (O.D. approximately 0.03 at 445 nm). Corresponding O.D. readings at 445 nm are: 1+ = 0.45, 2+ = 0.85, 3+ = 1.25, 4+ ≥ 2. For *E. coli*, fluorescence paralleled the generation of yellow color.

TABLE 1
Effect of Heterotrophic Bacteria on the Specificity of the Defined Substrate Technology

Species of HPC[b]	No Coliform	Visual Interpretation of the Defined Substrate Test[a] Species of Coliform (4 CFU/100 ml)			
		E. coli[c]	*K. pneumoniae*	*E. cloacae*	*C. freundii*
Pseudomonas maltophilia	Neg	4+/3+(>1)[d]	3+(>1)	4+(>1)	3+(>1)
Aeromonas hydrophilia	Neg	3+/3+(>1)	4+(>1)	4+(>1)	3+(>1)
Flavobacterium breve	Neg	3+/4+(>1)	3+(>1)	4+(>1)	2+(0.82)
Pseudomonas + Aeromonas	Neg	4+/4+(>1)	3+(>1)	4+(>1)	3+(>1)
Pseudomonas + Flavobacterium	Neg	4+/3+(>1)	3+(>1)	4+(>1)	4+(>1)
Aeromonas + Flavobacterium	Neg	3+/3+(>1)	4+(>1)	4+(>1)	3+(>1)
Pseudomonas + Aeromonas + Flavobacterium	Neg	3+/3+(>1)	3+(>1)	4+(>1)	3+(>1)

[a] At 24 hours' incubation at 35°C, ambient air

[b] CFU/ml: Pseudomonas, Aeromonas, Flavobacterium alone: 20,000; Pseudomonas + Flavobacterium and Pseudomonas + Aeromonas: 10,000 each; Pseudomonas + Aeromonas + Flavobacterium: 10,000 each

[c] Yellow/fluorescence

[d] O.D. reading at 445 nm

TABLE 2
Chlorine Injury of Coliform Species and Recovery by the Defined Substrate Technology

Species	% Injury[a]	% Recovery of Injured Coliforms[a]	
		TL Agar[b]	Defined Substrate
E. coli	80	93	86
K. pneumoniae	86	90	91
E. cloacae	82	88	88
C. freundii	76	92	87

[a]Average of three determinations
[b]Trypticase lactose agar

mixed with each of the species of total coliforms (at four total coliforms per 100 ml), visible color was detected well within the time frame of the test (Table 1). Color intensity was not appreciably different from the data presented in Fig. 1, in which the total coliforms were tested in pure culture. False-positive analyses were not noted at HPC concentrations as high as 20,000 per ml, even with species that contained the β-galactosidase system. In no instance did a yellow or fluorescent tube result unless one of the target microbes was present.

The defined substrate technology was able to detect the four isolates of injured coliforms; it recovered approximately the same percentage of injured bacteria as standard recovery agar (Table 2). Compared to uninjured coliforms, injured bacteria required more time to produce a positive defined substrate tube (Fig. 2). Each species was detected within the 24-hour time frame of the method, however. Final color intensity was weaker with injured as compared to uninjured bacteria. Once color production had been noted from the injured coliforms, subsequent yellow or fluorescence developed as rapidly as from uninjured bacteria (Fig. 2). It appeared that there was a delay in the lag phase of the injured bacteria in order for them to repair themselves. Once they had done so, they grew as well as normal bacteria.

DISCUSSION

The foundation for quantitative microbiology began when Kass, in 1956, described the association between 10^5 bacteria per ml of urine and true urinary tract infection. Clinical microbiology laboratories developed manual and automated means to perform these assays during the next two decades [28]. In the 1970s, work began on the means to identify microbes rapidly based on their constitutive enzymatic content [29] and to apply this information to enumerate a particular species from mixtures in clinical specimens [20,23,29,30]. The source of bacteria causing urinary tract infections and those used as indicators of microbial pollution are largely the same: the coliform group of *Enterobacteriaceae*. These bacteria include *E. coli, K. pneumoniae, E. cloacae,* and *C. freundii*. Therefore, it seemed possible that the defined substrate technology used to enumerate individual species or groups of bacteria from mixtures in urine could be applied to environmental microbiology.

The MTF and MF methods for the analysis of water for fecal pollution contain several inherent limitations that result in decreased sensitivity and specificity. Central to both methods is the inclusion of a broad-based protein source in the analysis media. This mixture contains a sufficient variety of nutrients to support a wide spectrum of microbial growth. Specificity in these media is achieved by including inhibitory

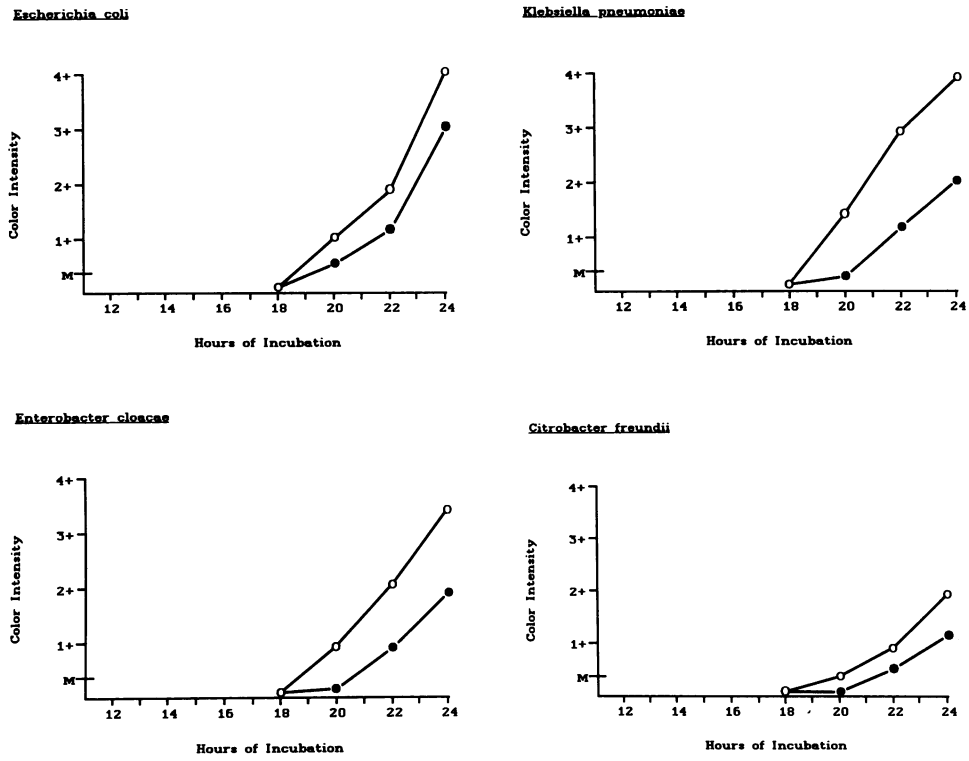

FIG. 2. Comparison of uninjured and injured coliforms in their ability to generate the ortho-nitrophenyl chromogen. Open circles represent uninjured bacteria and solid circles are the chlorine-damaged microbes. Readings are the same as those described for Fig. 1.

detergents and dyes such as sodium dodecyl sulfate and crystal violet; however, this specificity is only relative. The defined substrate technology achieves specificity in a different way. It includes a nitrogen source, ammonium sulfate, in a simple salt solution that enteric bacteria can use as their sole source of nitrogen. Instead of lactose, the defined substrate technology relies on ONPG (yellow) for total coliforms and MUG (fluorescence) for *E. coli*. The use of specific substrates allows the incorporation of chromogens with different colors to be used in the same analysis vessel and to enumerate simultaneously two classes of indicator bacteria: total coliforms for regulatory purposes and *E. coli* for public health information. Because of the increased inherent specificity of the defined substrate technology, one does not have to perform confirmed and completed tests, which can require two to four days. Most contamination of water distribution systems follows cross-connections or point-source events; these incidents generally result in the presence of high levels of bacteria as long as the contamination event continues. The ability of the defined substrate method to detect the point-source event eight to ten hours earlier than MTF or MF methods, which require at least 24 hours, can result in earlier remedial action and the potential prevention of disease.

The sensitivity of methods for water analysis must be able to detect one bacterium per 100 ml. The defined substrate technology met this goal both with pure cultures of environmental isolates and when the target microbe(s) were mixed with competing

heterotrophs in ratios as high as 2×10^6 to 1. In addition, ten raw water samples (Lake DeForest, New City, NY) were tested and also showed no inhibition of the total coliforms with heterotrophs [unpublished results]. In this small sample, the defined substrate method showed equal sensitivity and specificity to the *Standard Methods* MTF method [2].

It is known that heterotrophic bacteria can yield both false-positive and false-negative MTF and MF analyses. *Aeromonas* spp. are most commonly responsible for false-positive tests because many isolates can ferment lactose. In the defined substrate technology, however, *Aeromonas* concentrations as high as 20,000 per ml did not yield a positive within the time period of the test. One *Aeromonas hydrophilia* of twenty *Aeromonas* species tested did yield color at 32 hours of incubation, eight hours after the completion of a normal defined substrate analysis, at 20,000 per ml [unpublished results]. The inability of lactose-fermenting heterotrophs to generate a positive defined substrate analysis appears to be due to their inability to assimilate ammonium sulfate to induce the permease or galactosidase systems.

False-negative MTF and MF analyses can result because of HPC suppression of the target microbe(s). This suppression results from a combination of the competition for food and the release of inhibitory factors, such as bacteriocins, produced by heterotrophs. False-negative MTF and MF tests can occur at HPC concentrations as low as 500 per ml [9,31]. There was no HPC suppression with any of the four species of target microbes, even at HPC:total coliform ratios as high as 2×10^6:1. Therefore, the defined substrate technology demonstrated equal sensitivity and potentially better specificity than MTF and MF methods.

The treatment of water relies heavily on chlorine to eliminate microbial pollution. Chlorine may not only kill bacteria but may also sublethally injure them. Although the exact biochemical lesion is not known, it is thought that the microbe's nucleic acid is modified [32]. In order for the bacterium to grow it must repair its DNA, which requires time, energy, and appropriate environmental conditions. MTF and MF methods contain detergents and dyes which inhibit the repair of the cell. Because the defined substrate method does not contain inhibitors, and does contain sodium sulfite, which is known to aid in the synthesis of cell walls, it should permit repair of the injury. The defined substrate technology did grow injured coliforms with the number of recoverable CFU equal to the control. Compared to uninjured coliforms, there was a longer lag time until color was first noted. Once color production was observed, the subsequent increase in optical density over time was equal to that of the uninjured coliforms. Therefore, it appeared that the lag phase of injured coliforms was prolonged, reflecting the time needed for repair, but once the lesion was corrected the bacteria grew as well as their normal counterparts. Each of the species of coliforms demonstrated the same repair characteristics.

Although it is somewhat premature to compare the cost of the defined substrate method to that of conventional methods, it is generally conceded that a complete water analysis for total coliforms costs approximately $15 [33]. The defined substrate method should cost between $4.50 and $8.00. The major saving lies in its significantly reduced labor.

The defined substrate technology for the enumeration of target microbe(s) from a mixed bacterial population, originally devised for urine specimens, has been applied to environmental analysis. Utilizing bacteria isolated from a water supply source, it showed the necessary sensitivity, one CFU per 100 ml, to make it applicable for water

processing for regulatory purposes. It was not subject to false-positive and false-negative tests due to heterotrophic bacteria and, unlike MTF and MF methods currently in use, it did not require multi-day confirmatory and completed tests. The defined substrate technology also possessed the ability to enumerate two classes of indicator bacteria simultaneously: the total coliforms and *E. coli*. The method is easier to use than *Standard Methods* procedures [2] and has the potential to provide greater public health information at a lower cost.

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